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SELECTIVE TARGETING OF ANTIVIRAL AND IMMUNOMODULATING AGENTS IN THE TREATMENT OF ARENAVIRUS INFECTIONS

Annual Report

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A manuscript entitled "Therapeutic Efficacy of Liposome-Encapsulated Ribavirin and Muramyl Tripeptide in Experimental Infection with Influenza or Herpes Simplex Virus", has been accepted for publication in the Journal of Infectious Diseases and is now "In Press". (See Reverse Side)

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The studies presented in this report further document the ability of liposome-encapsulation to augment the immunopotentiating and therapeutic properties of the synthethic immunomodulator, muramyl tripeptide (MTP-PE). Encapsulation of MTP-PE into large negatively charged liposomes enhanced this drugs ability to activate peritoneal, alveolar, and liver macrophage antiviral functions.

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Supplementary Notes (Con't)

Some of the data in this report was presented at the V-International Conference on Comparative Virology, May 4-9, 1986, Alberta, Canada, and the International Symposium on Immunological Adjuvants and Modulators of Non-specific Resistance to Microbial Infections, June 30 - July 3, 1986, Columbia, Maryland.

Abstract (Con't)

Moreover, liposome-encapsulation of MTP-PE reduced virus replication in target organs and provided better protection in murine models of herpes simplex type 1 (HSV-1) pneumonitis and hepatitis. In contrast, uncapsulated MTP-PE was more effective than liposome-encapsulated MTP-PE in augmenting resistance to HSV-1 induced encephalitis; thus, free MTP-PE crossed the blood brain barrier and activated either the humoral and/or cellular components of CNS immunity. Liposome delivery of MTP-PE together with the broad spectrum antiviral ribavirin, was examined in guinea pigs lethally infected with Pichinde virus. Guinea pigs receiving either free or liposome-encapsulated ribavirin survived longer and had less virus in target organs than did untreated animals. Nonetheless, once ribavirin therapy was stopped, 80% of the animals died and virus could be detected in the spinal cord and brain. In contrast, 80% of the guinea pigs receiving intranasal instillation of liposome-encapsulated MTP-PE together with unencapsulated ribavirin survived. Animals receiving both drugs had reduced virus titers in the liver, lung, spleen, and adrenals, and no virus was detected in the central nervous system. The data confirm and extend our previous observations that liposomes can enhance the therapeutic potential of antiviral agents in those diseases in which the reticuloendothelial system is involved. In addition, the combination of both liposome-encapsulated MTP-PE and ribavirin appears to be highly effective in arenavirus infections in which both visceral organs and the central nervous system are involved.

SUMMARY

An important prerequisite for the therapeutic success with an antiviral agent is the ability to reach specific targets within the host and be maintained at therapeutic levels without resulting in host toxicity. Drug carries such as liposomes may be useful in this regard since these vehicles can be designed to deliver chemotherapeutic agents to infected target sites and thus avoid exposure of uninfected cells or organs. The data presented in this report were generated during the second year of a three year study evaluating the therapeutic potential of liposomes as carries for the selective targeting of antiviral and immunomodulating agents.

We have examined the therapeutic value of large negatively charged liposomes as vehicles for the targeted delivery of the broad spectrum antiviral, ribavirin, and the synthetic immunostimulant, muramyl tripeptide (MTP-PE), to primary sites of virus infection in a diseased host. The animal models of infection examined included mice infected with hepatic or encephalitic strains of herpes simplex type 1 (HSV-1), and guinea pigs infected with the arenavirus, Pichinde. Murine models of HSV-1 were selected because of this virus's predilection for the macrophage and dissemination to either the lung, liver or central nervous system (CNS) depending on the route (i.n.,i.v., or footpad) by which it is administered. Likewise, Pichinde virus infection of guinea pigs results in viral replication in macrophages and dissemination to the liver, spleen, lungs and adrenals early in infection, and spinal cord and brain later in infection. Moreover, the viral pathology observed in this animal model resembles that observed in human Lassa virus infections. Both liposome encapsulated and nonencapsulated MTP-PE and ribavirin were compared for their ability to reduce virus replication in primary target organs and protect against lethal HSV-1 and Pichnide virus infections. Treatment of mice with liposome-encapsulated MTP-PE was more effective than unencapsulated MTP-PE in activating alveolar and liver macrophage antiviral functions. Moreover, liposome-encapsulated MTP-PE was superior to free MTP-PE in reducing virus replication and in enhancing resistance to HSV-1 pneumonitis and hepatitis. In contrast unencapsulated MTP-PE readily crossed the blood/brain barrier, and was better than liposome encapsulated MTP-PE in augmenting resistance to HSV-1 induced encephalitis. Likewise, treatment of Pichnide virus infected guinea pigs with either free or liposome-encapsulated MTP-PE or ribavirin reduced virus titers in key target organs (i.e. liver, spleen, lung and adrenals) but did not prevent animals from developing a late CNS infection which ultimately resulted in death. However, intranasal instillation of both liposome-encapsulated MTP-PE and free ribavirin significantly reduced virus replication in all organs examined and enchanced long-term survival. The data presented in this report has resulted in the following conclusions:

1. Liposome-encapsulation significantly enhances the ability of the synthetic immunostimulant, MTP-PE, to augment macrophage antiviral functions. Moreover, because of their predilection for the reticuloendothelial system, liposomes provide a highly selective means by which immunostimulants can be delivered to both pulmonary and liver macrophages.

2. In viral infections of the CNS, liposome encapsulation of MTP-PE is not as effective as free drug in enhancing resistance and protection appears to result from passage of free drug through the blood/brain barrier. While the cellular and/or humoral mechanisms by which MTP-PE enhances CNS immunity are unknown, this observation has opened new avenues through which the therapy of viral encephalities may be approached.

3. Combination chemotherapy in which both MTP-PE and ribavirin are used together augments drug activity and provides a therapeutic approach in which both the early (visceral) and late (CNS) stages of arenavirus infections can be controlled.

4. The intranasal route of administration of either ribavirin or MTP-PE has proved to be highly effective in Pichinde and HSV-1 infections of the lung and CNS. While studies are still underway to more accurately define the therapeutic activity of these drugs following either i.v., i.p., or i.n. administration, it is apparent that the intranasal route of administration is highly effective in the treatment of arenaviurs infections.

FOREWORD

Citations of commerical organizations and trade names in this report do not consitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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I. PROBLEM UNDER INVESTIGATION

A number of clinically proven antivirals such as amantadine, iododeoxyuridine, adenine arabinoside, and acycloguanosine are currently in use; however, to the best of our knowlege, these drugs are of little value in the treatment of arenavirus infections. Several potentially useful antivirals (e.g., ribavirin and selenazole) possess a broad spectrum of activity against both DNA and RNA viruses (including arenaviruses) and are currently undergoing clinical evaluation. Clinical acceptance of these antivirals could be hastened by the development of delivery vehicles which would provide their sustained release in virus-infected target organs and avoid contact with non-infected tissues, thus, minimizing toxicity.

This study addresses the therapeutic value of liposomes for the targeted delivery of antiviral and immunomodulating agents to infected organs in a diseased host. More specifically we are examining both large multilamellar and small unilamellar liposomes for their ability to:

1) deliver immunostimulants and antivirals to the site(s) of primary virus replication, enhance cellular immunity, and reduce virus replication;

2) protect antiviral substances from the normal degradative and clearance mechanisms of the host during the journey from the site of inoculation to the site(s) of infection;

3) increase uptake and retention of antivirals in infected tissues;

4) reduce therapeutic dosages normally required to inhibit virus replication in infected organ and cellular sites; and

5) reduce the toxic effects observed with conventional modes of drug delivery.

II. BACKGROUND

Successful drug use in biology and medicine is often prejudiced by the failure of drugs that are otherwise active in vitro to act as efficiently in vivo. This is because in the living animal drugs must, as a rule, bypass or traverse organs, membranes, cells and molecules that stand between the site of administration and the site of action. In practice, however, drugs can be toxic to normal tissues, have limited or no access to the target and be prematurely excereted or inactivated. A number of antiviral agents (e.g., ribavirin, adenine arabinoside, phosphonacetic acid) are effective in vitro in preventing virus replication and/or cell death; however, their systemic use in man is limited by the induction of toxic effects which occur at dosages required to maintain effective drug concentrations in the infected organ. In particular, sustained treatment often results in leukopenia and subsequent immunosuppression which may affect the outcome of treatment since recovery from most viral infections also involves the cooperation of host immune responses. As in the case of ribavirin where dose limiting toxicity is the development of anemia (1,2), there is now growing optimism that such problems may be resolved with the use of carrier vehicles that will not only protect the nontarget environment from the drugs they carry but also deliver and facilitate their release at the site(s) in which they are needed.

During the past decade, a variety of carrier vehicles have been advocated for the selective targeting of antitumor drugs. Thus, there are numerous reports on the association of drugs such as anthracyclines, methotrexate, bleomycin, chlorambicin, and 1-B-D-arabinofuranosyl cytosine (cytosine arabinoside) with carriers such as DNA (3,4), liposomes (5,6), immunoglobulins (7,8), hormones (9,10) red blood cell ghosts (11) and other proteins (12,13) or polypeptides (14).
Most of these carriers have the ability to selectively interact with target cell surfaces and are subsequently endocytosed and transferred to the lysosomal compartment. Free drug is released intracellularly when the bond between the drug and the carrier is hydrolysed by lysosomal enzymes (15). In contrast, liposomes may sometimes deliver their contents directly to the cytoplasm following fusion with the target cell membrane (16). This mode of delivery is useful for drugs which are susceptible to lysosomal enzymes since membrane fusion is a mechanism through which contact with the lysosomal compartment of the cell can be bypassed.

In addition to the examples already cited, liposomes have also been used as carriers for i) the hepatic delivery of arsenicals in the treatment of leishmaniasis (17), ii) iododeoxyuridine in the treatment of herpes keratitis (18), and iii) amphotericin B in the treatment of Candida albicans (19), murine leishmaniasis (20), histoplasmosis (21), and cryptococcosis (22). The mechanism(s) by which liposomes enhanced the chemotherapeutic index of these drugs has not been defined; nonetheless, increased and prolonged tissue concentration of both iododeoxyuridine and amphotericin B in infected sites are most likely involved.

Our rational for the use of liposomes as carriers in the delivery of antivirals to virus infected tissues or organs in a diseased host is based on previous observations in which these macromolecular carriers provided a therapeutic advantage (23). Ribavirin (1-B-D-riboflulansyl-2, 4-triazole-3-carboxamide; ICN Pharmaseuticals) was selected as the prototype antiviral to be encapsulated because of its broad spectrum antiviral activity and relatively low toxicity.

The synthetic analog of muramyl dipeptide, muramyl tripeptide-phosphatidyl ethanolamine (MTP-PE) is produced by CIBA-GEIGY and was selected as the immunostimulant of choice because of its defined immunoenhancing properties, and potential for use in humans.

III. Experimental Approach

Our experimental approach during the second year of this three year study was designed with the following goals in mind:

1. Extend initial observations on liposome enhancement of MTP-PE immunostimulation with special emphasis on antiviral properties of liver and alveolar macrophages.

2. Examine the prophylactic and therapeutic activity of MTP-PE and ribavirin in murine models of HSV-1 hepatitis and encephalitis.

3. Establish both the MHA hamster and strain 13 guinea pig models of Pichinde virus infection, and determine the therapeutic value of liposome-encapsulated MTP-PE and/or ribavirin in these models.

A. Preparation of Liposomes

Large negatively charged multilamellar vesicles (MLV) containing phosphatidyl serine, phosphatidyl choline and cholesterol (3:7:1 ratio) were produced using the rehydration procedure described by Alving et al (17). This liposome design was selected because of its high encapsulation efficiency, uptake by the reticuloendothelial system, and localization in target organs such as the lung and liver following intravenous administration. Chemical analysis of liposomes was done by high performance liquid chromotography, while tissue distribution was performed by autoradiography. The prophylactic and therapeutic potential of liposome encapsulated ribavirin and MTP-PE was examined in C_3H/Hen mice infected with HSV-1, and in strain 13 guinea pigs or MHA hamsters infected with Pichinde virus (see below for complete description of virus models).

All animals received between 1.4 and 2.8mg phospholipid per dose either alone (sham liposome) or in combination with MTP-PE or ribavirin. A complete description of the procedures used to encapsulate either MTP-PE or ribavirin was presented in last year's Annual Report.

B. Animal Models

The following is a description of the experimental models of virus induced disease used to evaluate the therapeutic potential of liposome-encapsulated antivirals and/or immunostimulants. C_3H/Hen mice used in these studies were obtained from our breeding colony maintained at the University of South Carolina School of Medicine. This breeding facility rountinely screens mice for the presence of Sendai virus, mouse hepatitis, and other adventitious agents, and provides for the continous source of young (3-4 week old) mice used in the HSV-1 encephalitis model described below.

1. HSV-1 Hepatitis. The virus used in this model is the VR-3 strain of herpes simplex type 1 and was obtained from the laboratory of Dr. Andre Nahmias (Emory Univ., Atlanta GA.). Intravenous inoculation of 5-6 week old mice with $1x10^3$ p.f.u. results in death 6-9 days post-infection. The liver is the primary target organ, but by day 4 post-infection, the virus can be found in most visceral organs as well as the brain and spinal cord. Immunoperoxidase staining reveals massive antigen accumulation in the liver 48-72 hours after infection.

2. HSV-1 Encephalitis. The virus used in this model is a human isolate (MB strain) of type 1 herpes simplex virus obtained from Dr. Richard Whitley (Univ. Ala., Birmingham, AL). Footpad inoculation of four week old mice with $5x10^5$ p.f.u. results in virus replication in the sciatic nerve, spinal cord, adrenals and brain. Mice die of an encephalitis 7-10 days after inoculation. Immunoperoxidase staining of viral antigen in the spinal cord is present 3 days post-infection, while viral antigen and replication in the brain is evident by day 5 post-infection.

3. Pichnide Virus Infection of Strain 13 Guinea Pigs. Pichnide virus (ATCC strain AN4763) was obtained from Dr. Peter Jarhling (USAMRIID, Ft. Detrick, MD.) as a 10% spleen suspension. This virus had been passaged 11 times in guinea pigs prior to receipt and was designated 4763 GP-11. Our stock virus was prepared by inoculating strain 13 guinea pigs (Crest Caviary, Calif.) intraperitoneally with 10^4 p.f.u. of Jahrling's virus. At 7-days post-infection, spleens were removed and the virus plaqued on Vero cells. Clones were picked and this virus passed once again in strain 13 guinea pigs. Spleens were removed from infected animals and stored at - 80^0c as 10% homogenates. Our virus stocks have been designated spleen pass 13. Intraperitoneal or subcutaneous inoculation of 250-350 gram guinea pigs with 10^4 p.f.u. of this stock virus results in death 14-18 days post-infection. The virological and pathological findings which we have observed are basically the same as those reported by Jahrling et al (25). Replication initially occurs in the spleen and liver (day 4-7), and is followed by the lung and adrenals (day 5-9), and the spinal cord and brain (day 9-14). While the liver and spleen are heavily infected, much of the pathogical damage appears in the lung. Some inflammation at the hepatic portal triad with antigen deposition was observed; however, the most striking consistent finding was interstitial pneumonia with heavy antigen deposition as detected by immunoperoxidase staining.

4. Pichinde Virus Infection of MHA Hamsters. Pichinde virus (ATCC strain AN3739) was passaged 2 times in Vero cells to produce stock virus. Intraperitoneal inoculation of MHA hamsters (Charles River) with 10^3 p.f.u. of virus resulted in death 5-7 days after infection. In contrast to strain 13 guinea pigs, the liver of MHA hamsters appeared to be the primary site of virus replication.

Immunoperoxidase staining of liver sections revealed extensive hepatocyte destruction and heavy antigen deposition 4 days following infection. Lung involvement was not as extensive as in the guinea pig.

IV. RESULTS

A. Liposome Characterization

The size distribution of multilamellar liposome preparations containing either ribavirin or MTP-PE was determined by flow cytometry or laser microparticle analysis (Coulter). Approximately 70% of the liposome-encapsulated ribavirin particles produced by standardized procedures (see methods and last year's Annual Report) were 1-2 microns, 15% were 2-5 microns, 10% were 5-10 microns, and 5% were 10 microns or larger, the later were aggregates. Very similar size distribution appeared in MTP-PE liposome preparations. Smaller (80-100 nanometer) unilamellar liposomes were prepared using a commercially available dialysis instrument (Liposomat). Small unilamellar vesicles have been reported by others to remain in the circulation for prolonged periods and to eventually localize in the liver. Unfortunately, these small unilamellar particles were not stable in our hands and encapsulated drug (e.g. ribavirin) quickly leaked out; thus, their therapeutic value could not be accurately determined. High performance liquid chromotography (HPLC) procedures were developed to quantitate precise levels of ribavirin and MTP-PE per mole of lipid. Analysis of selected liposome preparations were routinely performed to verify drug dosage and encapsulation efficiency which averaged 15-22% for ribavirin and > 90% for MTP-PE. In addition these preparations were checked for the presence of endotoxin contamination using the Limulus amoebycte assay. Liposome preparations containing detectable endotoxin contamination (test sensitivity = 10 picograms) were discarded.

B. MTP-PE Induced Augementation of Peritoneal, Alveolar and Liver Macrophages

The data obtained during the first year of this study indicated that liposome-encapsulation of MTP-PE enhanced the ability of this drug to activate the phagocytic and bactericidal functions of peritoneal macrophages. More recent data obtained during the second year of this project has extended these findings to include augmentation of the antiviral functions of both alveolar and liver macrophages. Table 1 illustrates the enzyme contents, antiviral, and tumoricidal activities of peritoneal macrophages recovered from mice several days following i.p. inoculation of either free or liposome-encapsulated MTP-PE. Reductions in 5' nucleotidase and alkaline phosphodiesterase contents have been used by Morahan et al (26) as a marker of macrophage activation. The enzyme reductions observed in free MTP-PE, liposome-encapsulated MTP-PE, and C. parvum treatment groups correlates with the enhanced antiviral and tumoricidal functions observed.

Since it is commonly thought that activation of macrophages to the point in which they display tumoricidal activity is the best measure of a "fully activated" cell, we examined this function in alveolar macrophages lavaged from mice receiving intravenous inoculations of MTP-PE. As illustrated in Table 2, liposome-encapsulated MTP-PE was superior to free MTP-PE in activating alveolar macrophages. The degree of activation observed with liposome-encapsulated MTP-PE was never as high as that observed with C.parvum but was consistently higher than the activity observed with free MTP-PE.

In addition to our observations on peritoneal and alveolar macrophages, we also examined the effect of MTP-PE treatment on liver macrophages.

Since clearance of particles from the blood is a measure of RE function and in particular phagocytosis by liver macrophages, clearance of i.v. administered radiolabeled sheep red blood cells and localization of these cells in the liver was examined. In this clearance assay free MTP-PE was found to be as effective as liposome-encapsulated MTP-PE (Table 3). This findings was supported by flow cytometric analysis of phagocytic liver cells recovered from mice given fluroescent latex particles 48 hours after MTP-PE treatment (Figure 1). It should stressed that flow cytometry has been used in this instance to examine the phagocytic activity of individual cells. Liver cells recovered from mice receiving MTP-PE were more highly phagocytic than control cells.

C. Therapeutic Potential of Liposome-Encapsulated MTP-PE in HSV-1 Hepatitis

Mice, intravenoulsy inoculated with 10^4 p.f.u. of the VR strain of HSV-1, died 6-9 days post-infection. Protection (80% survival) was observed when mice were given both intravenous and intranasal therapy with liposome-encapsulated MTP-PE (Figure 2). In contrast, only 30% survival was observed in mice receiving similar therapy with free MTP-PE. Immunoperoxidase staining of livers from both free and liposome-encapsulated MTP-PE treatment groups revealed less viral antigen and less hepatocyte destruction in those animals receiving liposome-encapsulated MTP-PE. Our rationale for combining both the i.v.and i.n. treatment routes was based on our desire to deliver MTP-PE to visceral organs as well as CNS tissue (27) in this disseminated model of infection.

D. Therapeutic Potential of MTP-PE in HSV-1 Encephalitis

The enhanced effectiveness of liposome-encapsulated MTP-PE observed in HSV-1 hepatitis was similar to the observations reported for the therapy of HSV-1 induced pneumonitis in last year's Annual Report. This findings was not surprising considering the activation of liver macrophages; however, they are in contrast with the protection observed in HSV-1 encephalitis. When 4 week old mice were inoculated with $5x10^5$ p.f.u. of the MB strain of HSV-1, death occurred 6-9 days following infection. Significant protection against virus challenge was observed when mice were intranasally inoculated with free MTP-PE on days 0, 1 and 2 post-infection (Figure 3a and Table 4). No enhancement of this effect was observed in mice receiving prophylactic (e.g. day-2) or therapeutic intravenous inoculations of MTP-PE (Figure 3b). The superior protection afforded by free MTP-PE correlated with its ability to supress virus replication in spinal cords and adrenals of infected animals (Figure 4 and Table 5). It is interesting that even though free MTP-PE was able to prevent death and inhibit virus replication, latent virus was in some instances recovered from CNS tissue (Table 6).

E. Therapeutic Activity of MTP-PE in MHA Hamsters Infected with Pichinde Virus.

The virological and pathological features observed in MHA hamsters infected intraperitoneally with 5×10^3 p.f.u. of virus were similiar to those first described by Buchmeir et al (28). In this model, the liver is the primary site of virus replication and mice die 8-11 days after infection.

We have not been able to show any increase in survival rates following prophylactic or therapeutic MTP-PE treatment, but we have some evidence that MTP-PE administered prophylactically may prolong the mean survival time of infected animals (Table 7). Further work on this animal model has slowed since we have now established a more relevant model of human Lassa virus infection in strain 13 guinea pigs.

F. Therapy of Pichinde Virus Infected Guinea Pigs

Strain 13 guinea pigs (250-350 grams) infected intraperitoneally or subcutaneously with 1.0×10^4 p.f.u. of Pichnide virus (4763 GP pass 13) results in death 15-20 days following infection. Daily intraperitoneal administration of ribavirin (15mg/guinea pig) for 10 days and then every other day to day 19, prolongs the mean survival time and suppresses virus replication at several organ sites; however, most animals die by 30 days post-infection (Figure 5 and Tables 8 and 9). Ribavirin administered intranasally was as effective as i.p. ribavirin therapy. Similarly, liposome-encapsulated MTP-PE administered intranasally was effective in increasing the mean survival time of treated animals. Moreover, when ribavirin was used in combination with liposome-encapsulated MTP-PE, significant protection was observed (Figure 5). In addition, there was slightly less virus recovered in selected organs obtained from combination treatment groups than from animals receiving either treatment alone (Table 9). Thus a synergistic effect appeared when both ribavirin and MTP-PE were administered intranasally. These observations were based on five guinea pigs per treatment group. While the difference ($P<.05$) between combined therapy and monotherapy appeared to be significant, further studies with additional animals are needed.

V. CONCLUSIONS

1. Liposome-encapsulation significantly enhanced the ability of the synthetic immunostimulant, MTP-PE, to augment macrophage antiviral functions. Augmentation was observed in macrophages isolated from diverse anatomical sites (ie. lung, liver, peritoneum) confirming previous observations (1985, Annual Report) on the organ distribution of intravenously administered liposomes.

2. Because of their predilection for the reticuloendothelial system, liposomes provided a highly selective means by which immunostimulants were delivered to both pulmonary and liver macrophages, and thereby augmented nonspecific immunity to viral infections in which the lung and/or liver were primary targets of virus replication.

3. Viral infections of the central nervous system were not effectively treated with liposome-encapsulated MTP-PE due to the inability of liposomes to penetrate the blood/brain barrier. In contrast unencapsulated MTP-PE readily crossed this barrier and enhanced resistance to herpes encephalitis. The mechanism by which protection occured is unclear but presumably involved humoral and/or cellular components of CNS immunity.

4. Synergism between ribavirin and MTP-PE was observed following the intranasal instillation of both drugs in guinea pigs infected with Pichinde virus. Similiar effects were reported in last year's Annual Report where it was reported that both drugs administered together were more effective than either one alone in therapy of viral pneumonitis. Thus it appears that the virustatic drug, ribavirin, suppressed viral replication below a "critical point" in which the activated macrophage and/or its secreted products were able to eliminate remaining free virus and virus-infected cells.

VI. RECOMMENDATIONS

During the third year of this project (July 1986-June 1987), we would like to examine the therapeutic protocols established over the preceeding two years in the treatment of other arenavirus infections. Based on the data generated on the therapy of Pichinde virus infections,collaboration with investigators at USAMRIID on the therapy of Lassa and Junin virus infected guinea pigs should be initiated.

As described in last year's report, combination therapy employing both ribavirin and MTP-PE proved effective in preventing death due to viral pneumonia. Similar observations were documented in this year's report in which guinea pigs were infected with Pichinde virus. These observations suggest that a combined chemotherapeutic approach utilizing both the immunomodulatory activity of an immunostimulant and the virustatic or virucidal activity of an antiviral should be pursued. In particular, combinations in which several different immunostimulants (eg MTP-PE and/or poly I:C-LC) and antivirals (e.g. ribavirin and/or selenazole) either with or without liposome-encapsulation should be tested. Moreover since intranasal instillation of both ribavirin and MTP-PE appears promising, an attempt to develop aerosol delivery of these drugs should be made. The Aerobiology Division at USAMRIID should be contacted to help in the design and performance of these studies.

VII. LITERATURE CITED

1. Hillyard, I.W. In Ribavirin: A Broad Spectrum Antiviral Agent (R.A. Smith and W. Kirkpatrick, eds.). p.59-71, Academic Press, New York, (1980).

2. Ashwell, G., and Morell, A.G. Adv. Enzymol. 41, 99, (1974).

3. Trouet, A., Deprez-De Campeneere, D., and de Duve, C. Nature (London) New Biol. 239, 110-112, (1972).

4. Deprez-De Campeneere, D., and Trouet, A. Eur. J. Cancer 16, 981-;986. (1980).

5. Rustum, Y.M., Dave, C., Mayhew, E., and Papahadjopoulos, D. Cancer Res. 39, 1390-1395. (1979).

6. Gregiruadusm G. and Neerunjun, E.D. Biochem. Biophys. Res. Commun. 65, 537-544. (1975).

7. Ghose, T. and Blair, A.H. J. Natl. Cancer Inst. 61, 657-677. (1978).

8. Hurwitz, E., Levy, R., Maron, R., Wilchek, M., Arnon, R., and Sela, M. Cancer Res. 35, 1175-1181. (1975).

9. Kaneko, Y. Horm, Met. Res. 13, 110-114. (1981).

10. Varga, J.M., Asato, N., Lande, S., and Lerner, A.B. Nature (London) 267, 56-58. (1977)

11. Lynch, W.E., Sartiano, G.P., and Ghaffar, A. Amer. J. Hematol. 9, 249-259. (1980).

12. Kitao, T. and Hattori, K. Nature (London) 265, 81-82. (1977).

13. Monsigney, M., Kieda, C., Roche, A.C., nd Delmotte, F. FEBS Lett. 119, 181-186. (1980).

14. Ryser, H.J.-P., and Shen, W.-C Proc. Natl. Acad. Sci. USA 75, 3867-3870. (1978).

15. Trouet, A., Masquelier, M., Baurain, R., and Deprez-De Campeneere, D., Proc. Natl. Acad. Sci. USA 79, 626-629. (1982).

16. Poste, G. and Papahadjopoulos, D. Proc. Natl. Acad. Sci. USA 73, 1603-1607. (1976).

17. Alving, C.R., In Targeting of Drugs (G. Gregoriadis, J. Senior, and A. Trouet, eds.) p. 337-353, Plenum Publishing Corp., New York. (1982).

18. Smolin, G., Okumoto, M., Feiler, S., and Condon, D. Amer. J. Ophthal. 91, 220-225, (1981)

19. Lopez-Berestein, G., Mehta, R., Hopfer, R.I., Mills, K., Kasi I., Mehta, K., Fainstein, V., Luna, M., Hersh, F.M., and Juliano, R.J. Infec. Dis. 147, 939-945, (1981).

20. New, R.R.C., Chance, M.L., and Heath, S., J. Antimicrob. Chemother. 8, 371381, (1981).

21. Taylor, R.L. Amer. Rev. Respir. Dis. 125, 610-611, (1982).

22. Graybill, J.R., Craven, P.C., Taylor, R.L., Williams, D.M., Magee, W.E. J. Infect. Dis. 145, 748-752, (1982).

23. Kende, M. Alving, C.R., Rill, W., Swartz, G.M. and Canonico, P. Antimicrob. Agents and Chemo.27, 903-907, (1985).

24. Fidler, I.J., Raz, A., Fogler, W.E., Kirsh, R. Bugelski, P. and Poste, G. Cancer Re. 40, 4460-4464, (1980).

25. Jarhling, P.B., Hesse, R.A., Rhoderick, J.B., Elwell, M.A., and Moe, J.B. Infect. Immun. 32, 872-880, (1981).

26. Morahan, P.S., Edelson, P.J. and Gass, K. J. Immunol. 125: 1312-1317, (1986).

27. Fogler, W.F., Wade, R., Brundish, D.E., and Fidler, I.J., J. Immunol. 135: 1372-1377, (1985).

28. Buchmeier, M.J. and Rawls, W.E. Infect. Immun. 16:413 (1977).

TABLE 1 ENZYME CONTENTS AND ANTIVIRAL AND ANTITUMOR ACTIVITIES OF PERITONEAL MACROPHAGES

TREATMENT GROUP	ENZYME[a] ACTIVITY		ANTIVIRAL[b] ACTIVITY	TUMORICIDAL[c] ACTIVITY
	5'N	AP	$\log_{10}$ PFU/ml (% reduction)	(%cytotocicity)
PBS	27.3	14.7	8.04	22
FREE MTP-PE[d]	18.3	7.6	5.80 (99.42)[f]	41
SHAM LIPOSOME	0.9	18.6	5.36 (99.79)[f]	22
LIPOSOME ENCAPSULATED MTP-PE[d]	1.2	2.2	5.86 (99.34)[f]	40
C. PARVUM[e]	0.2	3.7	4.71 (99.95)[f]	72

a - 5' nucleotidase (5'N) and alkaline phosphodiesterase (AP) activities are presented as nanomoles/min/mg protein. Data provided by Dr. Page Morahan.

b - Virus titers from HSV-1 infected vero cells at macrophage to target cell ratio:1:1. Virus titers determined 72 hours post infection.

c - Isotope release from B-16 target cells at E:T=40:1.

d - 100 micrograms/mouse administered i.p. 2 days prior to harvest.

e - 1.4 mg/mouse administered ip 5 days prior to harvest.

f - Significantly different from PBS control.

TABLE 2 TUMORICIDAL ACTIVITY OF PULMONARY MACROPHAGES FOLLOWING MTP-PE TREATMENT[a]

TREATMENT GROUP	CELL ASSOCIATED COUNTS (AVERAGE CPM ± STD DEV)	%CYTOTOXICITY[b]
PBS	6498 ± 276	12
SHAM LIPOSOME	6110 ± 473	17
FREE MTP-PE	6112 ± 416	17
LIPOSOME ENCAPSULATED MTP-PE	5124 ± 735	30[c]
C. PARVUM[d]	3993 ± 263	46[c]
MAXIMUM TARGET CELL COUNTS	7340 ± 461	-

a - 100 μg i.v. 48 hours prior to lung lavage.
b - Effector:target ratio=20:1.
c - P .01 when compared to PBS control group.
d - 1.4 mg i.v. 120 hours prior to lung lavage.

TABLE 3 EFFECT OF MTP-PE TREATMENT ON RETICULOENDOTHELIAL FUNCTION[a]

TREATMENT	CLEARANCE FROM BLOOD (T/2)[b]	LOCALIZATION IN LIVER (CPM per mg. tissue)
PBS	4.3	146
FREE MTP-PE	1.8[c]	217[c]
SHAM-LIPSOME	2.52[c]	186[c]
LIPOSOME ENCAPSULATED MTP-PE	1.81[c,d]	215c
P. ACNES	2.04[c]	178

a - As indicated by clearance of chromium labeled sheep red blood cells from blood and its localization in liver.
b - Minutes taken to clear 50% of chromium labeled sheep red blood cells from circulation. Calculated from the slope of clearance (K value).
c - Significantly different from PBS treated group.
d - Significantly different from SHAM-LIP treated group.

TABLE 4 MEAN SURVIVAL TIMES OF MICE RECEIVING MTP-PE THERAPY FOR HSV-1 INDUCED ENCEPHALITIS

TREATMENT	TREATMENT SCHEDULE (days)	% SURVIVAL	MEAN SURVIVAL (DAYS)
CONTROL	-	0	8.50
FREE MTP-PE[a]	0, 1, 2	80[b]	13.50[b]
LIPOSOME ENCAP-SULATED MTP-PE[a]	0, 1, 2	50[b]	13.40[b]

a - 100 micrograms MTP-PE administered intravenously on the indicated days.

b _ $P < .05$ when compared to controls.

TABLE 5 INHIBITION OF HSV-1 REPLICATION FOLLOWING MTP-PE THERAPY

Treatment	Virus Titers			
	Spinal Cords		Adrenals	
	3 days p.i.	7 days p.i.	3 days p.i.	7 days p.i.
PBS	1.0×10^4	3.7×10^6	2.6×10^5	3.1×10^7
MTP-PE	0	3.8×10^4	5.0×10^3	0

a - 100μg/mouse i.v. on days 0, 1, 2 post-infection.
b - Log_{10} PFU/gram wet weight of tissue. Average titers from two randomly selected mice for each treatment group.

TABLE 6 Rescue of Latent Virus From MTP-PE Treated Survivors

Mouse #	Treatment[a]	Virus Titers[b] 7 days post infection			Co-cultivation[c] 31 days post infection		
		Adrenal	Spinal Cord	Brain	Adrenal	Spinal Cord	Brain
1	none	$2.0X10^7$	$3.3X10^6$	$8.0X10^5$	-mouse died-		
2	MTP-PE	0[d]	0	0	$3X10^2$	$7.5X10^2$	0
3	MPT-PE	0	0	0	0	0	0
4	MPT-PE	0	0	0	0	$8X10^2$	0

a - MTP-PE (100 μg/mouse) i.v. on the day of and 2 days post footpad infection with 10 LD_{50} of HSV-1.

b - Determined on vero cell monolayers; Log_{10} plaque forming units per gram wet tissue.

c - Minced tissue fragments added to vero cell monolayers and incubated for 10 days. Values represent plaque forming units per ml in culture supernatants.

d - No plaques detected with undiluted samples.

TABLE 7 MTP-PE Therapy of Pichinde Infected MHA Hamsters

Group	Survivors[c]	Mean Survival Time[a]	
		Prophylactic	Therapeutic
PBS	0/10	10.3	10.5
Sham Liposome	0/10	15.2	16.2
Liposome[b] MTP-PE	0/10	15.6	10.4
Free MTP-PE	0/10	21.6	12.0

a. Expressed as days post-infection. Prophylactic treatment includes drug 2 days prior to, on the day of and 2 days post-infection. Therapeutic treatment includes drug on the day of and days 1 and 2 post infection.
b. 150micrograms MTP-PE per dose was administered i.p.
c. Animals were challenged i.p.with 5 x 10^3 pfu of Pichinde virus.

Table 8. Pichinde Virus Titers in Selected Organs[a]

	i.p. Treatment Groups:			
	Control	Ribavirin[b]	MTP-PE[c]	Ribavirin+MTP-PE
Liver	1.0×10^7	6.4×10^4	4.0×10^7	3.0×10^5
Lung	9.0×10^8	2.7×10^6	4.0×10^7	8.6×10^6
Spleen	3.0×10^7	2.0×10^6	3.6×10^7	1.7×10^7
Adrenal	2.5×10^8	1.6×10^4	1.0×10^7	4.7×10^5
Spinal Cord	3.6×10^5	< 10	2.0×10^4	< 10
Brain	2.9×10^5	< 10	5.5×10^5	< 10

a. p.f.u./gm.wt. 14 days post-infection
b. 15mg/guinea pig i.p. every day for 10 days then every other day to day 19 post-infection.
c. 200µg/guinea pig i.p. day 0,2,4,6,8 & 10 post-infection.

Table 9. Pichinde Virus Titers in Selected Organs[a]

	i.n. Treatment Groups			
	Control	Ribavirin[b]	MTP-PE[c]	Ribavirin+MTP-PE
Liver	1.0×10^7	3.3×10^5	2.5×10^6	7.5×10^5
Lung	9.0×10^8	6.3×10^6	3.5×10^7	2.5×10^6
Spleen	3.0×10^7	6.0×10^5	5.5×10^5	3.5×10^5
Adrenal	2.5×10^4	3.5×10^4	5.5×10^6	7.5×10^3
Spinal Cord	3.6×10^4	< 10	7.5×10^3	< 10
Brain	2.9×10^5	< 10	6.0×10^3	< 10

a. p.f.u./gm.wt. 14 days post-infection.
b. 15mg/guinea pig i.n. day 0-10, and day 12,14,16, & 18 post-infection.
c. 200µg/guinea pig i.n. day 0,2,4,6,8 & 10 post-infection.

Figure 1. Cell Sorter Analysis of Liver Macrophages Following Phagocytosis of Fluorescent Latex Particles.

Mice were given intravenous injections of liposome-encapsulated MTP-PE (100μg/mouse) 48 hours prior to the i.v. inoculation of fluorescent latex particles. Livers were excised 18 hours later and macrophages isolated and analyzed in an EPICS IV Flow Cytometer. Movement to right of the scale (bright dots) represents increased numbers of fluorescent latex particles phagocytosed by each cell.

Figure 1

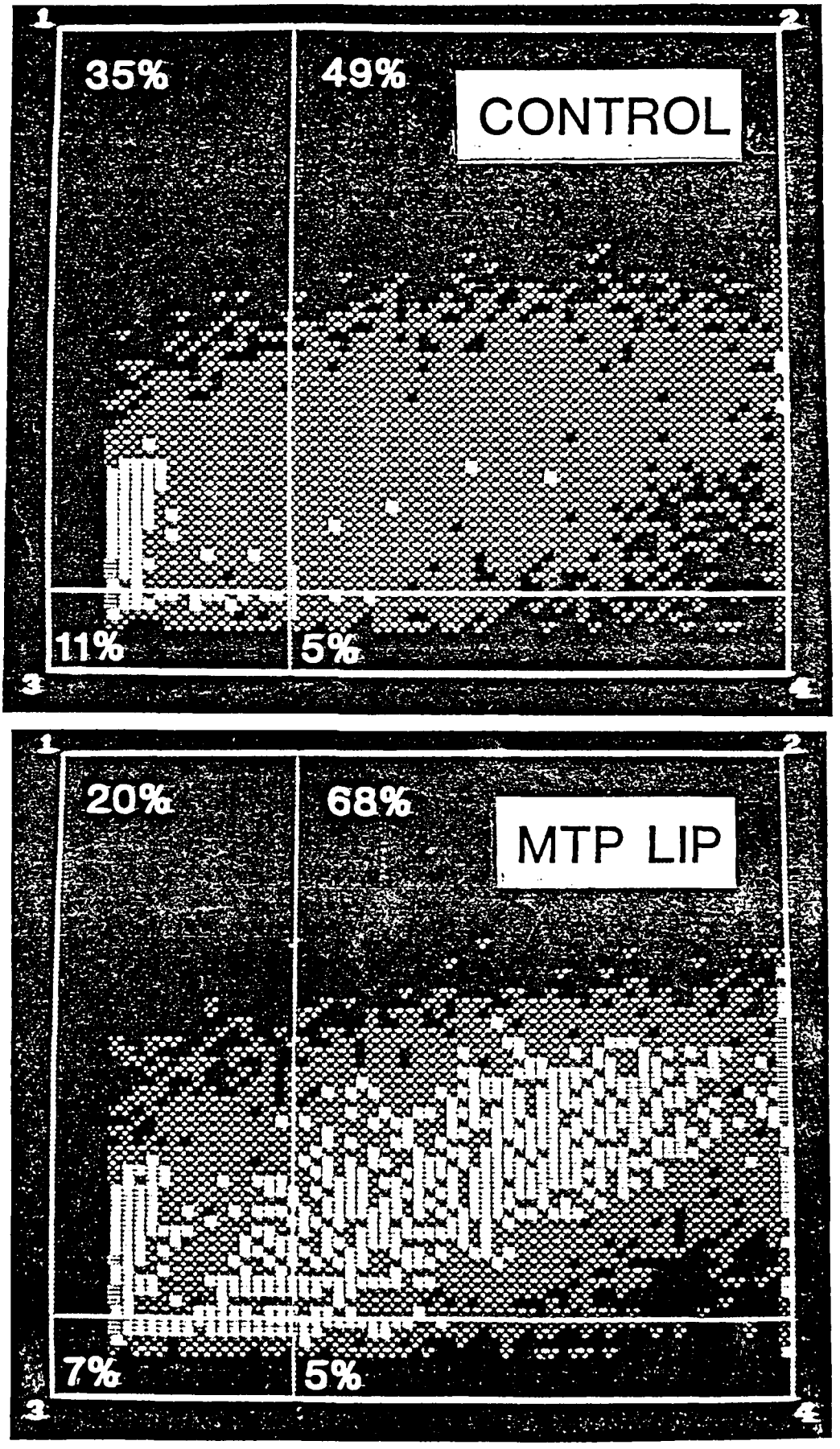
LOG RELATIVE FLUORESCENCE
1
2
35%
49%
CONTROL
11%
5%
3
4
1
2
20%
68%
MTP LIP
7%
5%
3
4

Figure 2. Therapeutic Activity of MTP-PE in Treatment of HSV-1 Hepatitis.

Five-six week old mice were inoculated intravenously with 10^4 p.f.u. of HSV-1 and treated on day 0 with 100μg (i.v.) of either liposome-encapsulated or free MTP-PE. This was followed by intranasal administration of free MTP-PE (100μg) on days 1 and 2 post-infection. Percent survival is based on 10 mice per group. $P<.05$ for liposome-encapsulated verses free MTP-PE

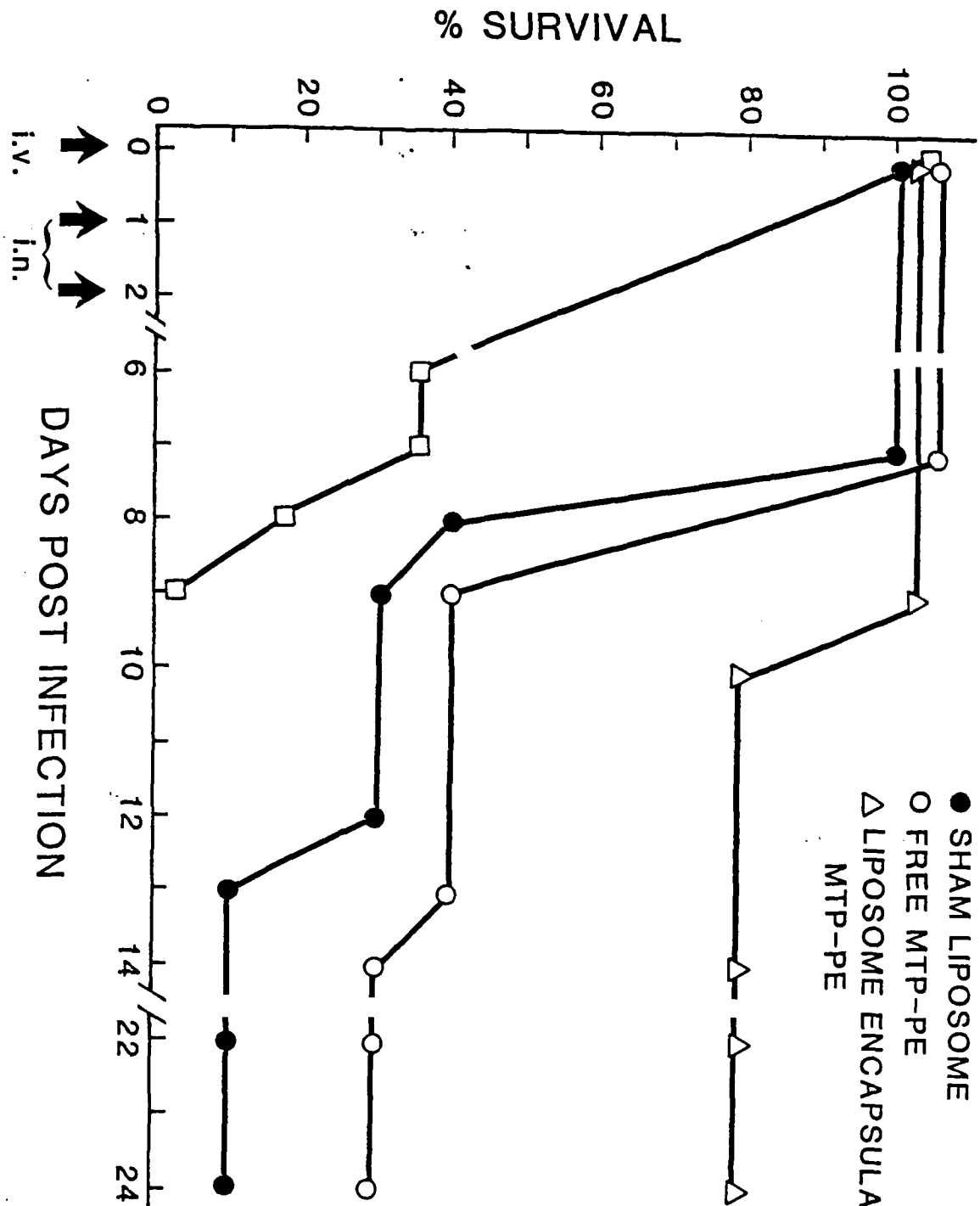
% SURVIVAL
0
20
40
60
80
100
0
1
2
6
8
10
12
14
22
24
DAYS POST INFECTION
i.v.
i.n.
□ VIRUS CONTROL - PBS
● SHAM LIPOSOME
○ FREE MTP-PE
△ LIPOSOME ENCAPSULATED MTP-PE

FIGURE 2

Figure 3a. Intranasal MTP-PE Therapy of HSV-1 Encephalitis.

Four week old mice were given $5x10^5$ p.f.u. of the MB strain of HSV-1 via footpad inoculation. Either free or liposome-encapsulated MTP-PE (100μg) was administered intranasally on days 0,1 and 2 post-infection. Percent survival was calculated from 10 mice per group. $P<.05$ for free verses liposome-encapuslated MTP-PE.

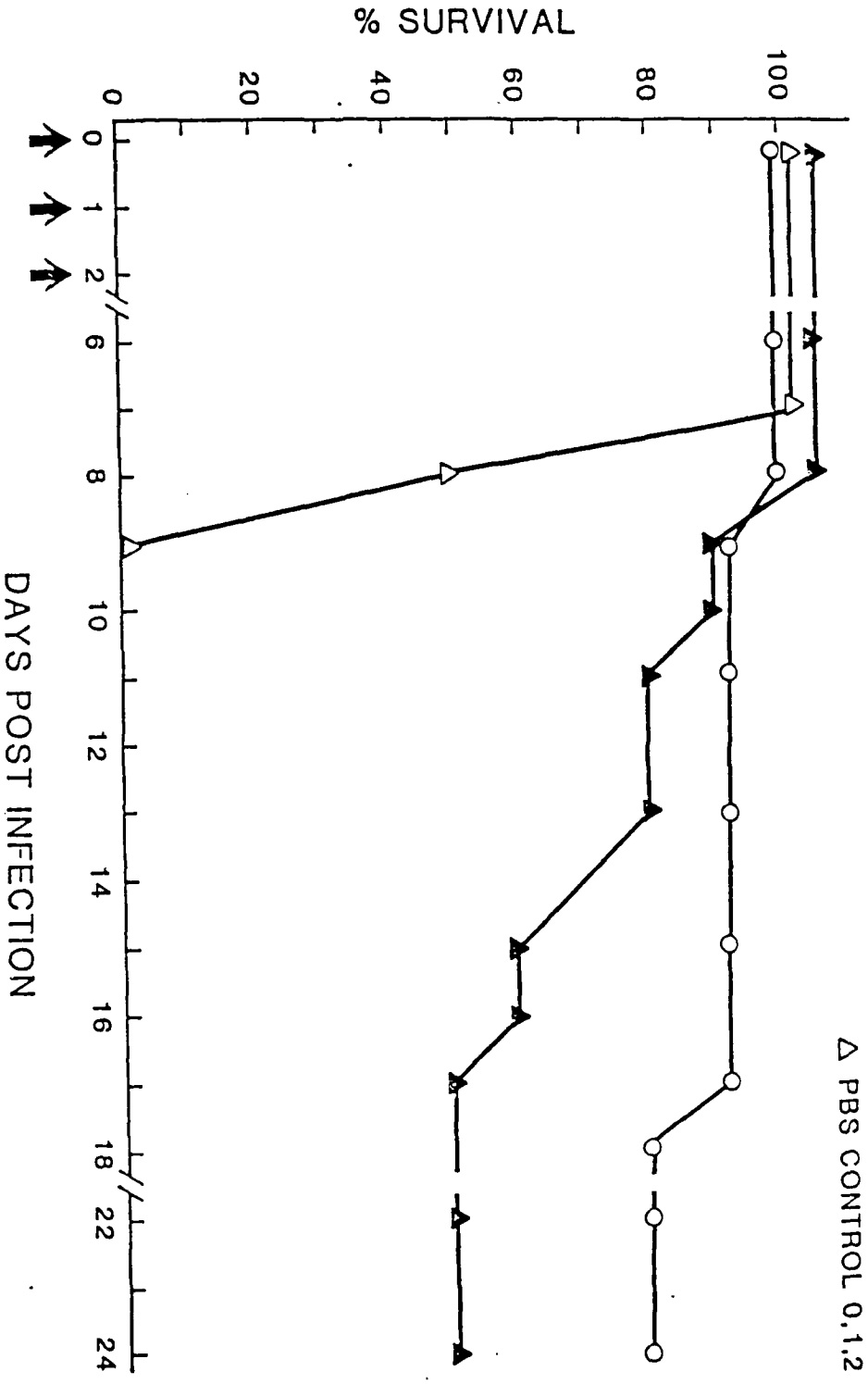
% SURVIVAL
0
20
40
60
80
100
0
1
2
6
8
10
12
14
16
18
22
24
DAYS POST INFECTION
▲ MTP LIP 0,1,2
○ FREE MTP 0,1,2
△ PBS CONTROL 0,1,2

FIGURE 3a

Figure 3b. Intravenous MTP-PE Therapy of HSV-1 Encephalitis

Four week old mice were inoculated as described in Figure 3a and intravenously administered free MTP-PE (100μg/mouse) on the days indicated. Ten mice per group were analyzed.

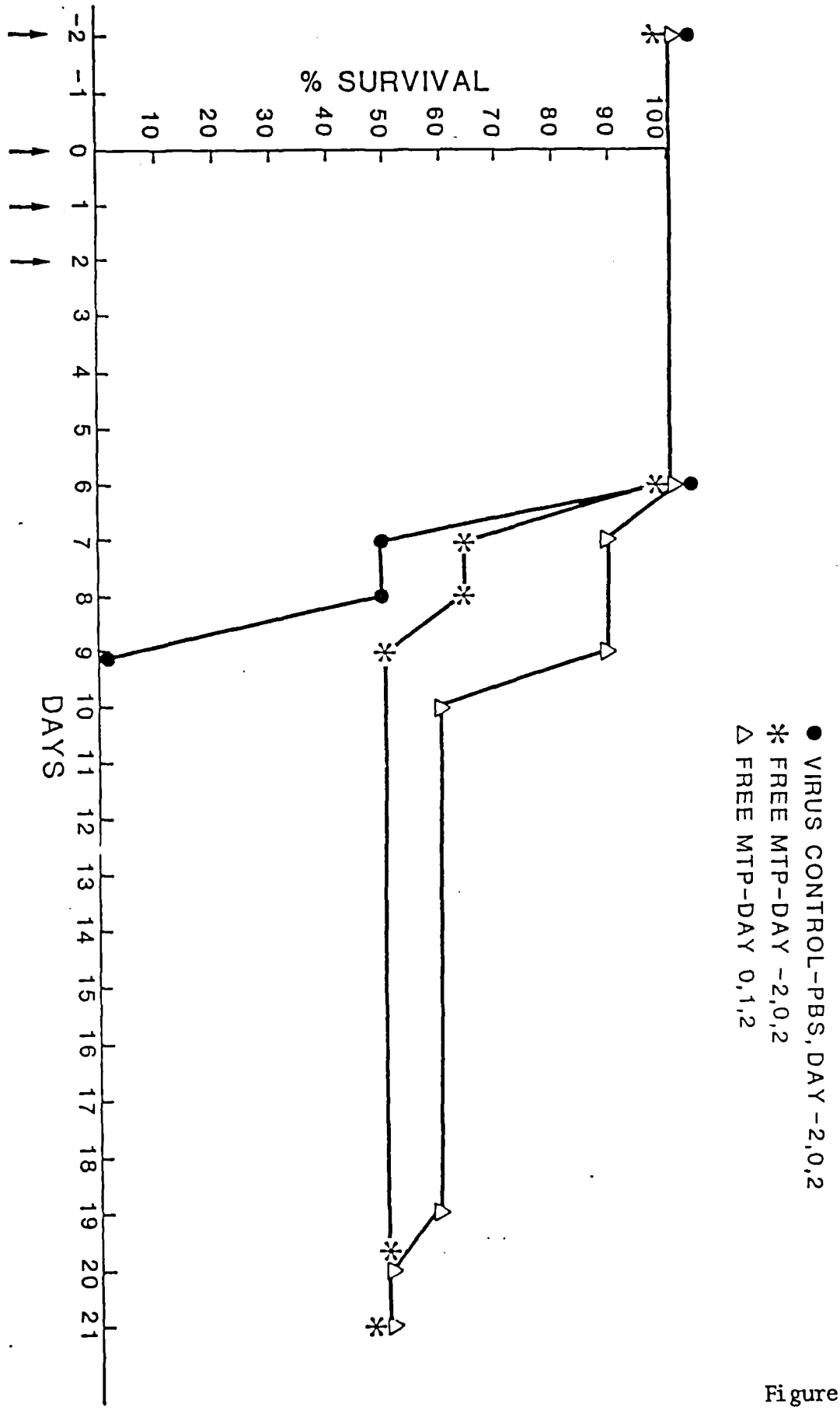
% SURVIVAL
100
90
80
70
60
50
40
30
20
10
-2
-1
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
DAYS
● VIRUS CONTROL-PBS, DAY -2,0,2
✱ FREE MTP-DAY -2,0,2
△ FREE MTP-DAY 0,1,2

Figure 3b

Figure 4. Virus Titers in Spinal Cords of Mice Receiving Intranasally Administered MTP-PE

Four week old mice were inoculated and treated with MTP-PE (100μg/mouse) as described in Figure 3a. Spinal cords were removed and infectious virus determined by plaquing on Vero cells.

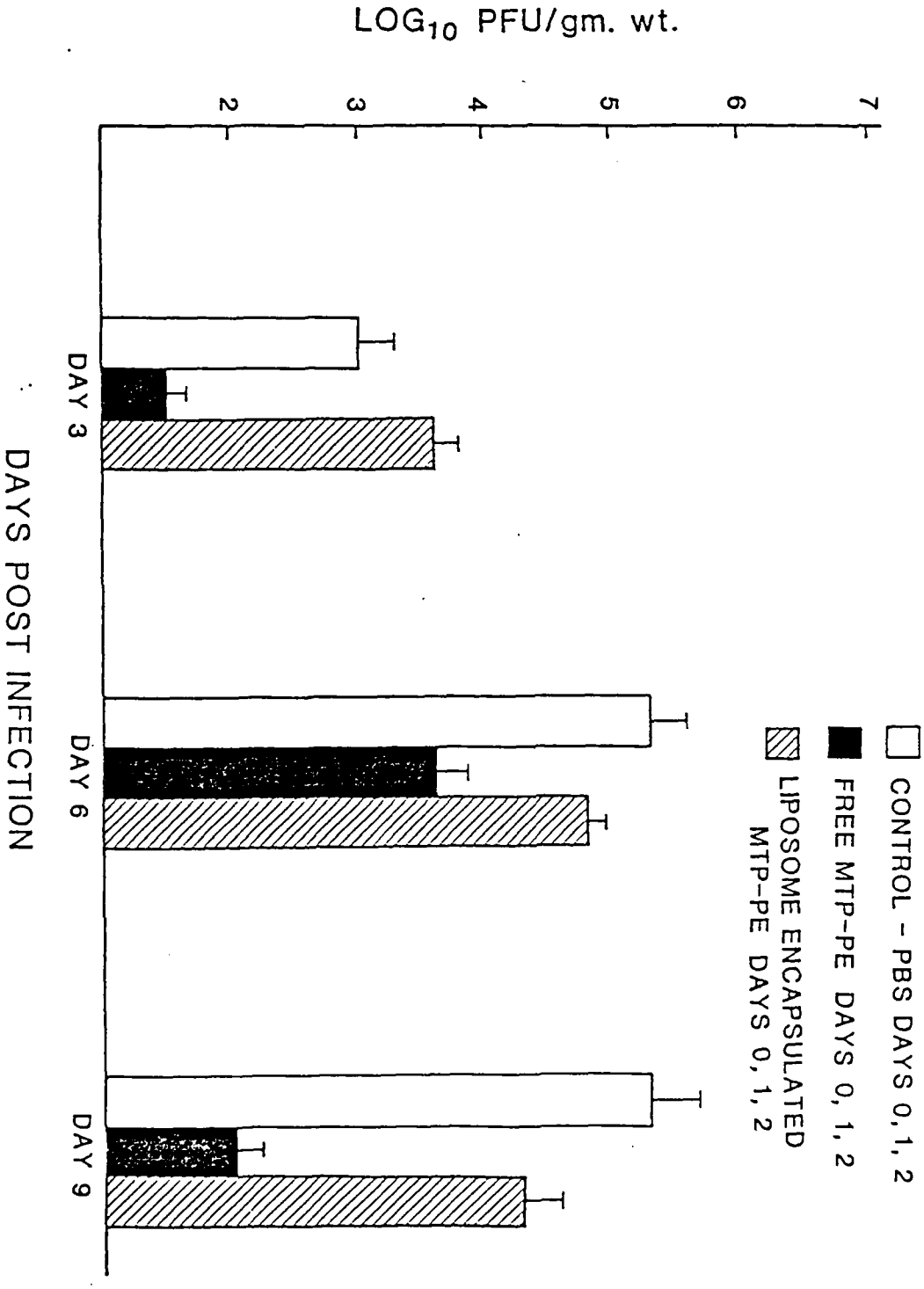
LOG10 PFU/gm. wt.
2
3
4
5
6
7
DAY 3
DAY 6
DAY 9
DAYS POST INFECTION
CONTROL - PBS DAYS 0, 1, 2
FREE MTP-PE DAYS 0, 1, 2
LIPOSOME ENCAPSULATED MTP-PE DAYS 0, 1, 2

FIGURE 4

Figure 5. Ribavirin and MTP-PE Therapy of Pichinde Virus Infected Guinea Pigs

Strain 13 guinea pigs (female 300-350 grams) were inoculated intraperitoneally with $1x10^4$ p.f.u. of Pichinde virus (AN 4763 GP-pass 13) and treated with ribavirin (15mg) and/or MTP-PE (200μg) via intraperitoneally or intranasal administration. See Tables 8 and 9 for complete description of drug dosage and scheduling. Five guinea pigs per treatment group were examined. $P<.05$ for combined i.n. therapy (ribavirin + MTP) verses monotherapy with either ribavirin or MTP.

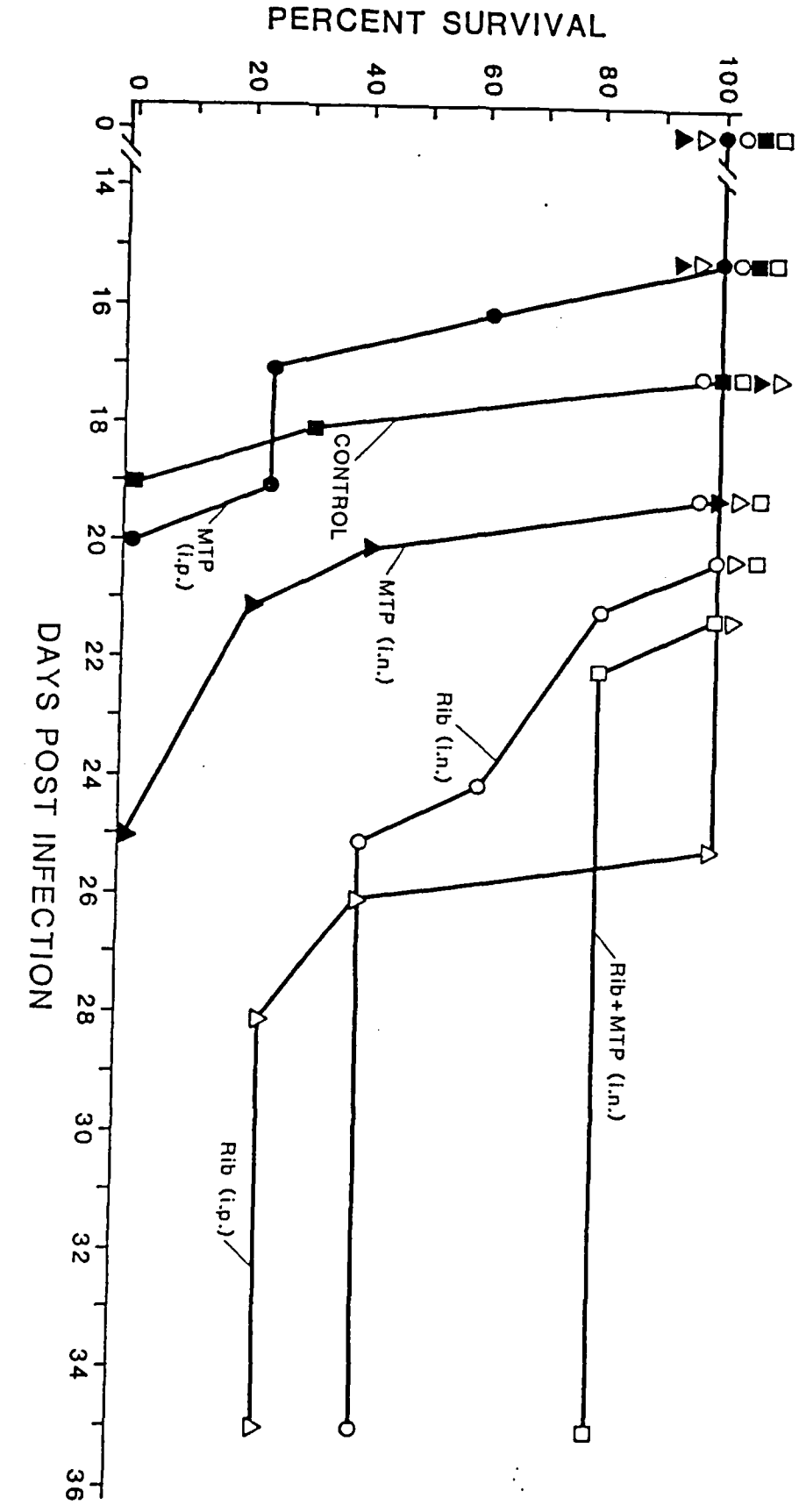
PERCENT SURVIVAL
0
20
40
60
80
100
0
14
16
18
20
22
24
26
28
30
32
34
36
DAYS POST INFECTION
CONTROL
MTP (i.p.)
MTP (i.n.)
Rib (i.n.)
Rib+MTP (i.n.)
Rib (i.p.)

Figure 5

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